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ARTICLE

Buckwheat (*Fagopyrum esculentum* Moench) FeMT3 Gene in Heavy Metal Stress: Protective Role of the Protein and Inducibility of the Promoter Region under Cu^{2+} and Cd^{2+} Treatments

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The protective role in vivo of buckwheat metallothionein type 3 (FeMT3) during metal stress and the responsiveness of its promoter to metal ions were examined. Increased tolerance to heavy metals of FeMT3 producing *Escherichia coli* and cup1$^{\Delta}$ yeast cells was detected. The defensive ability of buckwheat MT3 during Cd and Cu stresses was also demonstrated in *Nicotiana debneyii* leaves transiently expressing FeMT3. In contrast to phytochelatins, the cytoplasmatic localization of FeMT3 was not altered under heavy metal stress. Functional analysis of the corresponding promoter region revealed extremely high inducibility upon Cu^{2+} and Cd^{2+} treatments. The confirmed defense ability of FeMT3 protein in vivo and the great responsiveness of its promoter during heavy metal exposure make this gene a suitable candidate for biotechnological applications.

KEYWORDS: Buckwheat (*Fagopyrum esculentum* Moench); heavy metal tolerance; metallothionein; overexpression

INTRODUCTION

Contamination of soil and water with toxic chemicals is a very serious environmental and agricultural problem. Heavy metals are among the most harmful pollutants, greatly reducing the survival and yield of many commercially important plant species. Some of them, like Cu, Mn, and Fe, are essential for plant growth, serving as cofactors of important enzymes, but when present in high concentrations, these transition metals stimulate the generation of reactive oxygen species (ROS) by the Fenton reaction. Others, such as Cd, Pb, and Hg, do not have any known functions as nutrients and harm cells by blocking essential functional groups or by displacing necessary metal ions from biomolecules (*1*).

One effective, environmentally nondestructive, and cheap soil remediation method is phytoextraction (*2*). Metal toxicity in cultivated plants is usually connected with low soil pH, as acidity increases the availability of metals that may be abundant in polluted soils. Common buckwheat (*Fagopyrum esculentum* Moench) is well-known as an aluminum (Al) accumulator (*3*) and can also concentrate lead (Pb) in the shoot and especially in the leaf. Buckwheat is also known as a Cu and Zn accumulator and is able to tolerate high concentrations of these metals (*4*). This species has the potential to be a beneficial hyperaccumulator because of its short generation time and ability to grow in severe environments and on acid soils (*5*). Improvement of plants by genetic engineering, that is, by modifying characteristics like metal uptake, transport, and accumulation, opens up new possibilities for phytoremediation (*2*). Overproduction of metallochelating molecules such as phytochelatins and metallothioneins can greatly increase the metal accumulation capacity of plants (*6*). Also, the use of metal-induced promoters could have a great impact on biotechnological perspectives (*7*).

Our interest in buckwheat is primarily related to the potential biotechnological use of specific genes coding for proteins involved in metal homeostasis. One of these is the gene for metallothionein type 3, *FeMT3*.

Metallothioneins (MTs) are low molecular mass (4−10 kDa) proteins that bind metal ions, such as Zn^{2+}, Cu^{2+}, or Cd^{2+}, via the thiol groups of their cysteine residues (*8*). All MTs identified from plants so far belong to class II and have been further grouped into four types according to Cys residue distribution. It has been demonstrated that different types of MTs exhibit distinct and overlapping functions in maintaining the homeostasis of essential transition metals, detoxification of toxic metals, and protection against intercellular oxidative stress (*9−11*). Data on

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plant MT promoters are very scarce, and only a few promoters are characterized in detail. Most of these analyses focused mainly on determination of the tissue expression pattern, while some revealed inducibility of MT promoters by various stress factors including metal ions (*12*).

Previously, in our laboratory, two cDNA clones coding for MT3-like protein were isolated from the cDNA library of developing buckwheat seeds [GeneBank accession no. DQ681063 (pFeMT3.5 clone) and DQ681064 (pFeMT3.10 clone)]. RNA gel blot analyses confirmed the presence of the corresponding mRNAs in buckwheat seeds, roots, and leaves. Changes in MT transcript levels during development and in response to different stress conditions especially to Zn^{2+} and Cu^{2+} were also detected (*13*). The corresponding genomic clone FeMT4.1 (GeneBank accession no. AY361956), containing 71 bp of the 5′ UTR and 569 bp of the 5′ regulatory region, was isolated. Functional promoter analysis in transgenic plants revealed strong activity in pollen and leaves but a much weaker one in roots (*14*).

In this paper, we present results concerning the protective role of FeMT3 protein in vivo during metal stress as well as the responsiveness of its promoter to metal ions, suggesting an important role of the FeMT3 gene in the metal tolerance traits of common buckwheat.

MATERIALS AND METHODS

Functional Promoter Analysis. The FeMT3 promoter construct, −569::GUS, was created, and the obtained vector was used for stable *Agrobacterium tumefaciens*-mediated transformation of tobacco in vitro as described previously (*14*). T1 and T2 seeds were selected on a medium supplemented with kanamycin (100 mg/L). Surviving T2 seedlings were checked by polymerase chain reaction (PCR), grown to maturity, and used for $CdCl_2$ and $CuSO_4$ treatments.

Ten independent T2 transgenic tobacco *Nicotiana tabacum* Havana lines were treated with heavy metals. Aboveground parts of 4 week old plants, grown in the greenhouse, were placed in half-strength MS salts with or without 100 μM $CdCl_2$ or 50 μM $CuSO_4$ for 1 or 2 days. The GUS activity of the treated leaves was compared to that of leaves of transgenic plants kept in MS1/2 solution. Untransformed tobacco plants were used as the negative control. The fluorimetric GUS assay was performed as described in ref *15*.

cDNA Synthesis and Real-Time Reverse Transcription (RT)-PCR. Aboveground parts of buckwheat plants grown in a greenhouse were put in MS1/2 solution supplemented with 100 μM and 300 μM $CdCl_2$ or 10, 50, and 100 μM $CuSO_4$ solutions for 5 h or 1 day. RNA was isolated using the Rneasy Plant Mini Kit. Prior to cDNA synthesis, contaminating DNA was removed from the RNA samples using Ambion DNA-free Dnase Treatment and Removal Reagents. First strand cDNA was synthesized from 1 μg of RNA with M-MuLV reverse transcriptase (Biolabs) and random hexamer primers (Applied Biosystem) according to the manufacturer's instructions. The cDNA was diluted 1:5 with nuclease-free water and used for real-time PCR with primers specific for *FeMT3* (cDNA DQ681064): MT10F (5′-GGGGTCGTGTGAAACTAATGATAGGTC-3′) and MT10R (5′-GAACATACACAGAGAAGGGGGAAGG-3′). Forward (MT10F) and reverse (MT10R) primers were designed to anneal to coding and 3′ untranslated regions, respectively. Expression was normalized against histone H3 gene expression using the primer pairs (H3F 5′-GAAATTCGCAAGTACCAGAAGAG-3′ H3R 5′-CCAACAAGGTATGCCTCAGC-3′). Reactions were done as described in ref *16*. A serial 2-fold dilution of cDNA derived from control leaves was used as a standard curve to calculate amplification efficiency for *FeMT3* and histone *H3* primers. PCR efficiencies were 1.85 and 1.92 for the *FeMT3* and *H3* genes, respectively. Each reaction was performed in triplicate, and the specificity of amplification products was confirmed by melting curve analysis.

Cloning Using Gateway Technology. Gateway technology (Invitrogen) was employed for cloning as described in the user manual, with pDONR201 as the entry vector and suitable destination vectors.

Expression and Purification of FeMT3-GST Fusion Protein. The coding region of the cDNA pFeMT3.10 clone (GeneBank accession no. DQ681064) was cloned downstream of the His and GST tags within the pETG-30A expressing vector, using GATEWAY cloning technology (Invitrogen). The primers, DM1 (TATTTTCAGGGCATGTCGTCCAACTGCGGAAG), DM2 (AGAAAGCTGGGTCTCAATGGCAACCGCATGAGCAG), DM3 (GGGGACAAGTTTGTACAAAAAAGCAGGCTCTGAGAATCTTTATTTTCAGGGC), and DM4 (GGGGACCACTTTGTACAAGAAAGCTGGGT) with Gateway-compatible attB overhangs were employed for two-step PCR. *Escherichia coli* BL21 cells transformed with the obtained pETG-30A-FeMT3 plasmid were used for expression of FeMT3 fusion protein. Expression was induced by the addition of IPTG to 1 mM for 3 h at 30 °C in the presence of 500 μM $CuSO_4$. Cells were centrifuged, suspended in phosphate-buffered saline (PBS), and lysed by sonication in the presence of 1 mM DTT to prevent protein oxidation. After sonication, cellular debris was pelleted by centrifugation (15 min at 10000*g*), and the fusion protein GST-FeMT3 was isolated from the supernatant by affinity chromatography using Glutathione-Sepharose 4B (Amersham Pharmacia). Obtained proteins were analyzed by 12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE).

Immunoblot Analysis. Mouse Anti-His HRP monoclonal antibodies (Qiagen) were used for immunodetection. All immunodetection steps were performed as described by ref *16*.

Bacterial Growth of GST::FeMT3-Expressing *E. coli* Cells in the Presence of Cadmium. The tolerance of untransformed or transformed *E. coli* cells to cadmium in the growth medium was tested using *E. coli* BL21 harboring pETG-30A-FeMT3 or pGEX, expressing GST-FeMT3 fusion protein or GST, respectively. Starter cultures of bacteria were grown overnight and used at 1:100 dilution. Protein expression was induced by the addition of IPTG to 1 mM, with or without 0.3 mM $CdCl_2$. Cultures were shaken at 200 rpm/30 °C, and bacterial growth was monitored up to 22 h by measurements at A_{600}.

Yeast Functional Complementation Assays. The copper-sensitive *Saccharomyces cerevisiae* strain, referred to hereafter as cup1$^{\Delta}$:DTY4 (*MATa*, *leu2-3*, *112his3D1*, *trp1-1*, *ura3-50*, *gal1 cup1::URA3*), was transformed by introducing the p424-FeMT3 construct and p424 empty vector as described in ref *17*. Yeasts were grown in SC-Trp-Ura medium at 30 °C and 220 rpm to OD_{600} = 0.5. Three 10-fold dilutions were performed, and 3 μL of each dilution was spotted on SC plates supplemented with 75 μM $CuSO_4$ and 3.5 μM $CdCl_2$. The DTY4 yeast strain containing p424 vector was grown in SC medium and used as a negative control. Plates were incubated for 3 days at 30 °C and photographed.

Transient Tobacco Transformation and Metal Treatments. The coding region as well as the 5′ UTR and 3′ UTR of the cDNA pFeMT3.10 clone (DQ681064) were introduced downstream of the CaMV35S promoter within the pB2GW7,0 expressing vector, by GATEWAY cloning technology (Invitrogen), using the primers DM5 (GGGGACAAGTTTGTACAAAAAAGCAGGCTGAACATCAAAGCAAAATCACTTCTTACTTC) and DM6 (GGGGACCACTTTGTACAAGAAAGCTGGGTGTACAATAGAAACATCATTAGTCC). The obtained pB2GW7,0-FeMT3 vector was introduced into C58C1 strain *Agrobacterium* by electroporation.

For transient overexpression of FeMT3, *Nicotiana debneyii* leaves were chosen because their robustness and large size make them suitable for infiltration and stress treatments. Left halves of *N. debneyii* leaves were transformed by the syringe infiltration method using *Agrobacterium* C58C1 carrying the pB2GW7,0-FeMT3 vector, while the right halves, which served as controls, were infiltrated with C58C1 without the vector. *Agrobacterium* overnight cultures were grown to an OD_{600} of 1 and then centrifuged at 3000 rpm/room temperature/25 min, resuspended in water to OD_{600} 0.8 and infiltrated. Three days after transformation, overexpression was checked by isolation of RT-PCR. After isolation of RNA and DNase treatment (described above), first strand cDNA synthesis was carried out in 25 μL of reaction mixture containing 0.4 μM MT10F/R or H3F/R primers, 2.5 mM dNTPs each, and 1 U of Taq DNA Polymerase (Fermentas). PCR amplification was programmed for 30 cycles, with each cycle consisting of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min in a Biometra T1 thermalcycler. The transformation efficiency was also verified by real-time RT-PCR using the same primer pairs. Transformed leaves were excised, and their stems were placed in half-strength MS salts solution containing 50 or 100 μM $CuSO_4$ or 300 μM $CdCl_2$. On the second and third day of treatment, the leaves were photographed, and portions

were taken for chlorophyll and lipid peroxidation measurements, carried out as described in refs *18* and *19*.

CLSM Analysis of Transient FeMT3::EYFP Expression in Plant Cells. For monitoring FeMT3 cellular localization, *N. debneyii* cells were transiently transformed with FeMT3-EYFP fusion protein. The coding region as well as the 3′ UTR of the cDNA pFeMT3.10 clone were introduced downstream of the EYFP coding region within the pB7WGY2,0 destination vector, by GATEWAY cloning technology, utilizing primers DM7 (GGGGACAAGTTTGTACAAAAAAGCAG-GCTCAATGTCGTCCAACTGCGGAAG) and DM6 (GGGGACCA-CTTTGTACAAGAAAGCTGGGTGTACAATAGAAACATCATTA-GTCC). The obtained pB2GW7,0-FeMT3 vector was introduced into *Agrobacterium* C58C1 strain by electroporation. *A. tumefaciens* cells were taken up either in water or in 50 μM $CuSO_4$ or 50 μM $CdCl_2$ solutions and injected into leaves. Three to six days after transformation, cells were analyzed by confocal laser scanning microscopy (CLSM) using LSM 510 (Zeiss, Jena, Germany) with the following settings: excitation at 488 nm and emission at 505−530 nm band pass for EYFP and EGFP; excitation at 543 nm and emission at 560 nm long pass for chlorophyll autofluorescence.

Statistical Analysis. The results were analyzed by Student's *t* test using SPSS statistical software. Because the obtained values of the quantitative GUS assay did not show normal distribution, the nonparametric Mann−Whitney test and median values were used for comparison of different groups of samples. For both tests, p values < 0.05 were considered significant.

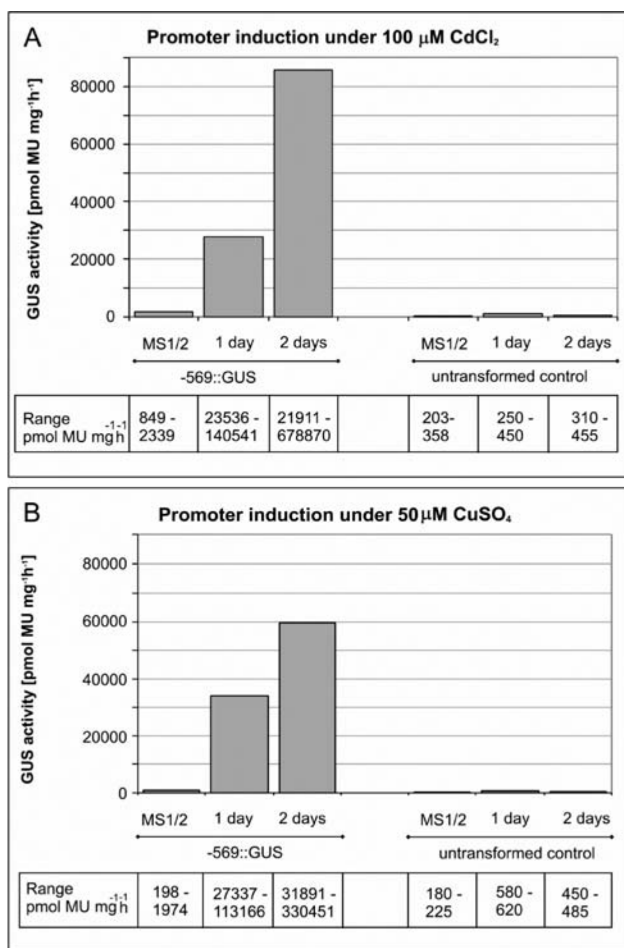

Figure 1. Fluorimetric GUS assay of leaves of T2 transgenic tobacco plants harboring the −569::GUS construct, in response to different metal stress treatments: (**A**) 100 μM $CdCl_2$ and (**B**) 50 μM $CuSO_4$. Values are the medians obtained for 10−20 independent transgenic lines. GUS activities of −569::GUS transgenic plants under treatments were significantly different from that of transgenic plants kept in MS1/2 solution ($p < 0.05$). GUS activities of untransformed plants under treatments were not significantly different from that of untransformed plants kept in MS1/2 solution ($p > 0.05$).

RESULTS

***FeMT3* Promoter Inducibility in Response to Cd^{2+} and Cu^{2+} Ions.** Plant MT gene expression was found to be influenced by various biotic and abiotic stimuli, the most important being metal ions (*8*). Computer-assisted analysis indicated four putative MREs (metal responsive elements) highly similar to the MRE core sequence (TGCRCNC) (*7*) from −418 to −424, −435 to −441, −451 to −457, and −485 to −491 bp upstream of the transcription start site within the *FeMT3* promoter region (*20*). Therefore, we tested the responsiveness of the *FeMT3* promoter construct −569::GUS to Cd^{2+} and Cu^{2+}. The *FeMT3* promoter region of 4 week old transgenic T2 tobacco plants was highly induced by the applied metal treatment after 1 day, showing 16.8- and 36.8-fold increases in activity in response to 100 μM $CdCl_2$ and 50 μM $CuSO_4$, respectively. After 2 days of treatment, the promoter activity was enhanced even more, up to 51.7- and 64.5-fold, when compared with control plants kept in MS1/2 solution (**Figure 1**).

Metal-Induced Transcription of FeMT3. As previously reported, two cDNA clones of the MT3 type, differing in 3′ UTRs, were isolated from the developing buckwheat seed cDNA library (*21*). Both clones comprised a 189 bp long coding region encoding for a 62 aa long polypeptide. Deduced amino acid sequence alignment showed that FeMT3 shared high homology with many type 3 MTs from other plant species. The greatest homology was revealed with MT3-like protein from *Carica papaya* (**Figure 2**). The N terminus showed greater homology to other MT3 members than the more variable C terminus. The FeMT3 C terminus contains six Cys consistent with the MT type 3 pattern ($CxCxxxCxCxxCxC_{(0-2)}x$) and one extra Cys at position 61.

Real-time PCR was used to investigate the influence Cu and Cd on the transcription level of *FeMT3* in buckwheat leaves at different time points, employing gene-specific primers. The *FeMT3* transcription level increased 1.6-, 1.35-, and 2.24-fold with 10, 50, and 100 μM $CuSO_4$, respectively, after 5 h of treatment. During the same time, 100 and 300 μM $CdCl_2$ treatments resulted in 1.94- and 1.9-fold increases, while after

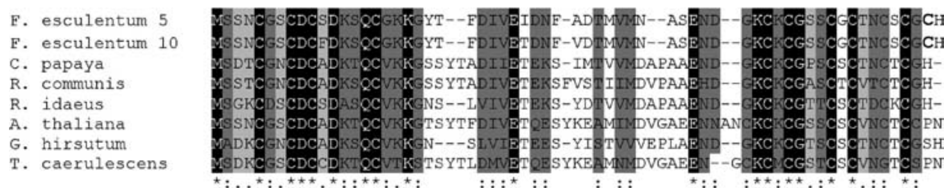

Figure 2. Sequence alignment of the predicted protein sequence of FeMT3 with sequences from representative plants [GeneBank codes: *F. esculentum* clone 5 (DQ681063) and clone 10 (DQ681064), *Carica papaya* (YO8322), *Ricinus communis* (EQ973975), *Rubus idaeus* (AJ224146), *Arabidopsis thaliana* (AF013959), *Gossypium hirsutum* (AY857933), and *Thlaspi caerulescens* (AY531114)]. Sequences were aligned by the ClustalW program. Identical amino acids are labeled with black boxes, highly similar amino acids are labeled with dark gray boxes, and less similar amino acids are labeled with gray boxes.

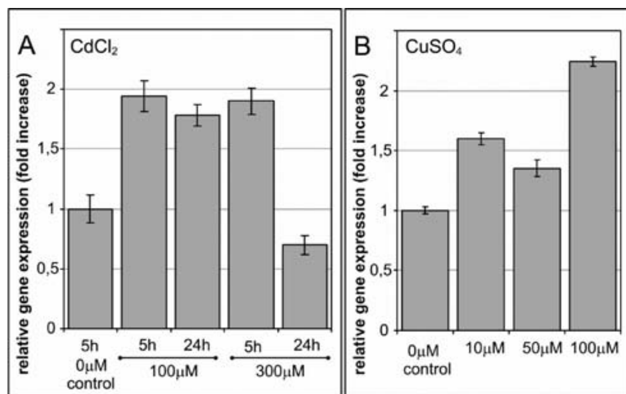

Figure 3. Real-time PCR analysis of FeMT3 gene expression in buckwheat leaves sampled during metal stress treatments: (**A**) $CdCl_2$ and (**B**) $CuSO_4$ (treatment duration 5 h). The means were generated from three independent measurements, and the bars indicate standard errors.

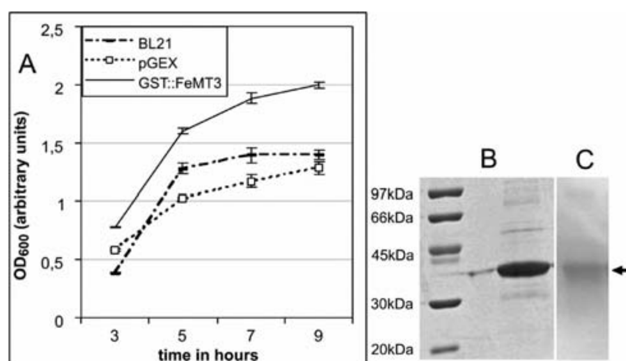

Figure 4. Heavy metal tolerance in transformed *E. coli*. (**A**) Growth curve of untransformed BL21 *E. coli* cells or BL21 cells expressing GST-FeMT3 (pETG-30A-FeMT3) or GST (pGEX) in 300 μM Cd-supplemented LB medium. Results are means $\pm$ SDs of three independent measurements. (**B**) SDS-PAGE of GST-FeMT3 fusion protein after purification by affinity chromatography using Glutathione-Sepharose 4B (Amersham Pharmacia). (**C**) Immunodetection of the recombinant GST-FeMT3 fusion protein.

1 day there was a transcript level decrease (0.69-fold) with the higher $CdCl_2$ concentration (**Figure 3A,B**).

Efficient Expression of Functional FeMT3 as a GST Fusion Protein in *E. coli* and Complementation Assay in Cup1$^{\Delta}$ Yeast. Recombinant FeMT3 tagged with GST was expressed in *E. coli* BL21 cells, purified on glutathione-Sepharose-4B columns, and visualized after 12% SDS-PAGE. The fused protein expressed in vitro at high levels was predominantly found in the soluble fraction. The presence of the FeMT3-GST fusion protein enabled the bacterial cells to grow in the presence of Cd at a significantly higher rate than cells expressing GST alone (**Figure 4**).

To test whether the expression of FeMT3 could provide protection against copper toxicity, Cu-sensitive yeast cells DTY4 were transformed with FeMT3. A functional copperthionein (CUP1) gene in *S. cerevisiae* is essential for prevention of copper-mediated cytotoxicity but is dispensable for cell growth in the absence of exogenous copper. The Cu tolerance threshold is reduced to 75 μM $CuSO_4$ in the cup1$^{\Delta}$ strain (cup1::URA3), which has no functional copies of CUP1 (*22*). Cup1Δ cells were transformed with p424-FeMT3 and plated on complete synthetic medium (SC), lacking Trp and Ura (SC-Trp-Ura) containing 75 μM $CuSO_4$ and 3.5 μM $CdCl_2$. The growth of p424 cup1$^{\Delta}$ cells was inhibited by $CuSO_4$, but those cells carrying p424-FeMT3

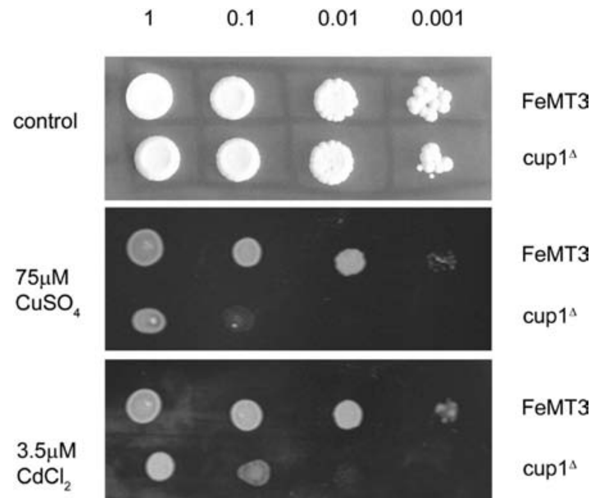

Figure 5. Yeast functional complementation assays. The CUP1-null (cup1$^{\Delta}$) cells were transformed with control p424 vector and p424-FeMT3 and grown to midlog phase. Ten-fold serial dilutions were made three times, and 3 μL of each was spotted on SC medium plates, supplemented or not supplemented with metals. Plates were incubated for 3 days at 30 °C and photographed.

were able to grow similarly to yeast cells plated on metal-free SC medium (**Figure 5**).

Influence of FeMT3 Overexpression on Metal Stress Tolerance in Planta. To examine if FeMT3 participates in cell protection in planta, *N. debneyii* leaves transiently overexpressing FeMT3 were treated with metal solutions and examined for stress tolerance. Left halves of the leaves were transformed with FeMT3 overexpression construct, while right halves of the same leaves were infiltrated with C58C1 *Agrobacterium* strain without the construct. Three days after infiltration with *Agrobacterium* C58C1 strain with or without the FeMT3 overexpression construct, parts of the leaves were excised, and their transformation was checked by RT-PCR (**Figure 6A**). The transformation efficiency was also verified by real-time RT-PCR. The *FeMT3* transcript level was 4.9−18.4-fold higher than histone *H3* transcript level in FeMT3 transformed tissue, while in mock-transformed tissue, FeMT3 amplification was not detected. After treatment with 300 μM $CdCl_2$ or 50 and 100 μM $CuSO_4$ for 3 days, the regions overexpressing FeMT3 exhibited a much higher ability to withstand the stress as compared to the mock-transformed regions. Thus, the control halves lost much more chlorophyll and developed a yellow color faster than the halves overexpressing FeMT3 (**Figures 6B−E** and **7**). Lipid peroxidation levels were also much lower in the parts overexpressing MT, indicating slower development of cell damage when compared to the control (**Figure 8**).

Cytoplasmatic Localization of FeMT3. Several recent studies revealed the exclusive cytoplasmatic localization of plant MTs, both in untreated and in metal ion-treated cells (*10*, *23*). We checked the location of FeMT3 and whether it is translocated upon stress treatment or not. **Figure 9** shows that in *N. debneyii* leaves transiently expressed FeMT3::EYFP was cytoplasmatically localized in both untreated and $CdCl_2$-treated *N. debneyii* leaves. The same localization was seen when $CuSO_4$ treatment was applied (data not shown).

DISCUSSION

As previously mentioned, buckwheat has the potential to be a useful hyperaccumulator, particularly for Al and Pb (*3*, *5*). This plant possesses relatively high biomass productivity, tolerance to

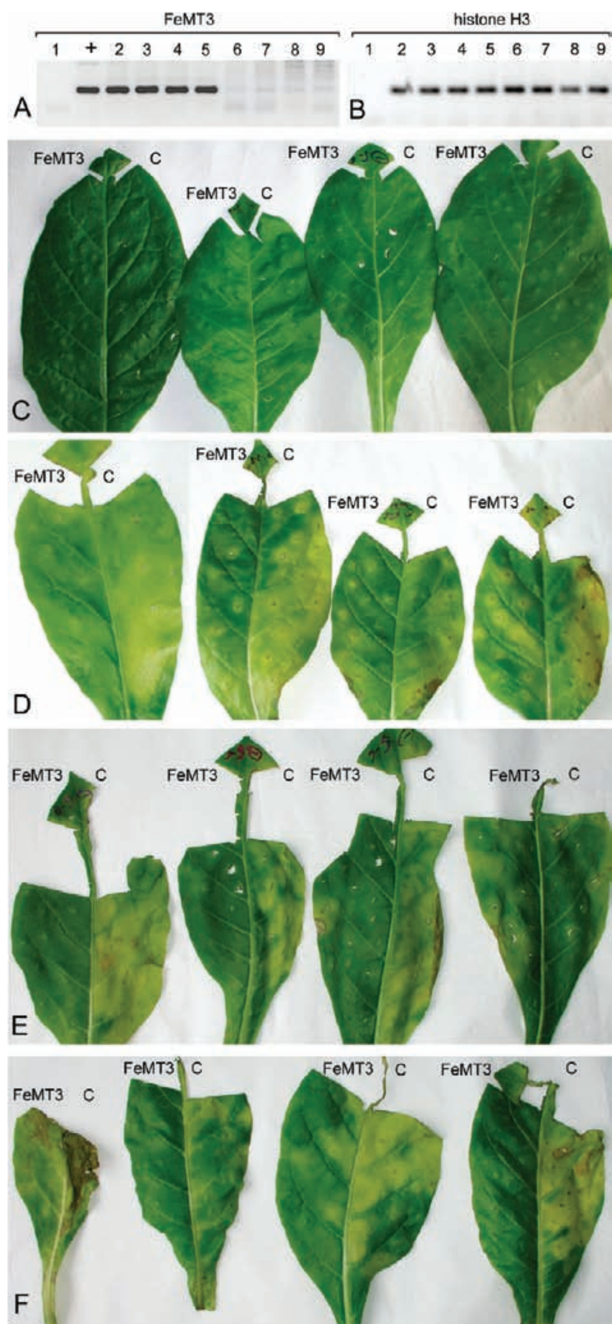

Figure 6. Effect of heavy metal treatments on transformed *N. debneyii* leaves. The left halves of the leaves were transiently overexpressing FeMT3, while the right halves were mock transformed using *Agrobacterium* C58C1 strain. Three days after agroinfiltration, parts of the leaves were excised and used for RNA isolation and RT-PCR confirmation of leaf transformation. RT-PCR was performed for each experiment replicate and *FeMT3* was found to be expressed in all tested leaves. Representative gel electrophoresis is shown. (**A**) RT-PCR using MT10F/R primers specific for FeMT3.10 cDNA clone and (**B**) RT-PCR using H3F/R primers specific for histone H3 gene. Numbers of lines in **A** and **B**: 1, H_2O; +, pB2GW7,0-FeMT3 vector; 2−5, cDNAs from leaf regions transformed with pB2GW7,0-FeMT3; and 6−9, cDNAs from leaf regions mock transformed using C58C1 *Agrobacterium* strain. *N. debneyii* leaves after 3 days of treatment in solutions of (**C**) MS 1/2 or MS1/2 supplemented with (**D**) 300 μM $CdCl_2$, (**E**) 50 μM $CuSO_4$, and (**F**) 100 μM $CuSO_4$. The experiments were repeated three times (a total of 60 leaves) with similar results.

unfavorable environmental factors, a wide distribution, and the ability to flourish in acidic and almost sterile soils (*5*). Considering

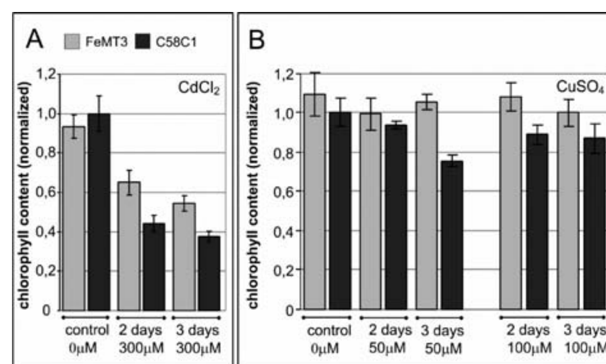

Figure 7. Total chlorophyll content of the transformed *N. debneyii* leaves under (**A**) 300 μM $CdCl_2$ and (**B**) 50 and 100 μM $CuSO_4$. Results are means ± SDs of three independent measurements. The values are normalized with the level of the mock transformed control in MS1/2. *p* values for chlorophyll content comparisons of the FeMT3 overexpressing to the mock transformed tissues were all <0.05 (significantly different) except for control (0 μM, that is, MS1/2) and 50 μM/2 days $CuSO_4$ treatments, which were >0.05 (not significantly different).

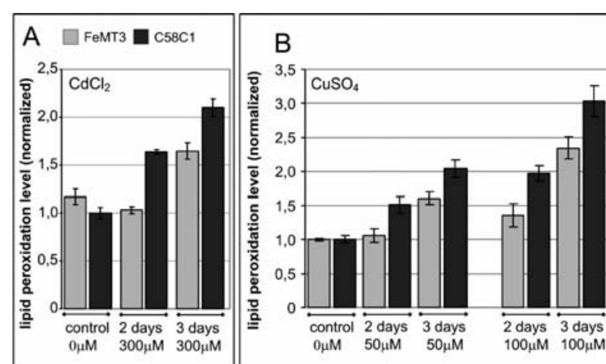

Figure 8. Lipid peroxidation level of the transformed *N. debneyii* leaves under (**A**) 300 μM $CdCl_2$ and (**B**) 50 and 100 μM $CuSO_4$. Results are means ± SDs of three independent measurements. The values are normalized with the level of the mock transformed control in MS1/2. *p* values for lipid peroxidation level comparisons of the FeMT3 overexpressing to the mock transformed tissues were all <0.05 (significantly different) except for control (0 μM, that is, MS1/2) treatment, which was >0.05 (not significantly different).

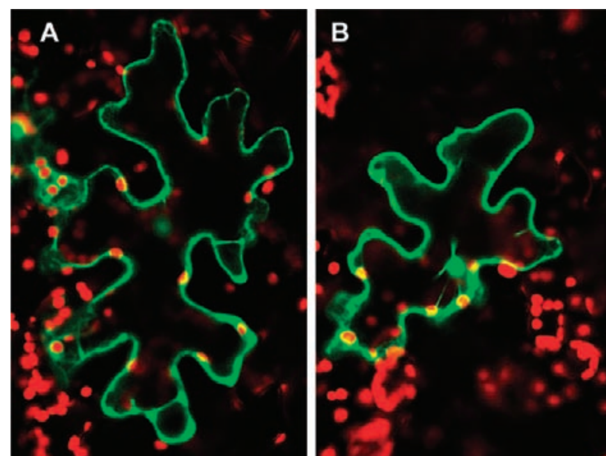

Figure 9. Cytoplasmatic localization of EYFP::FeMT3 transiently overexpressed in *N. debneyii* leaves: (**A**) before and (**B**) after 300 μM $CdCl_2$ treatment. Transformation of cells with a cytosolic EGFP control construct (pBinAR-EGFP) (*23*) gave identical distribution (not shown), confirming the exclusive cytosolic localization of FeMT3.

our previous findings concerning FeMT3, and bearing in mind the general properties of the MT protein and gene suitable for biotechnological applications, we decided to carefully investigate the ability of the buckwheat *MT3* gene to protect plant cells under heavy metal stress.

***FeMT3* Promoter Inducibility in Response to Cd^{2+} and Cu^{2+} Ions and Metal Induced Transcription of FeMT3.** The expression of MT genes has been characterized in many different tissues (*8*) and under various stress and environmental conditions (*9*, *10*, *24*). On the other hand, functional promoter analyses are very scarce. Most of them were focused mainly on determination of the tissue expression pattern, while some revealed MT promoter inducibility by various stress factors including metal ions. The Cu^{2+}-inducible promoter activity was detected for types 1, 2, and 3 MT genes in *Arabidopsis* (*25*), as well as for the rice *ricMT* promoter, also up-regulated by Zn^{2+} ions (*26*). To our knowledge, Cd^{2+} inducibility has not been reported so far for any of the isolated plant MT promoters.

We have already shown that Cu and Zn ions stimulate FeMT3 expression in leaves. Thus, expression was significantly enhanced in the early stage of seed development in response to Zn ions, but after this stage, no influence of Zn ions was detected. In this paper, we have extended these findings regarding the possible role of *FeMT3* in protecting plant cells from damage by heavy metals.

The presence of several putative MRE-like elements identified in the 5′ upstream region of the *FeMT3* gene correlates with its extremely high inducibility by heavy metals. Therefore, the *FeMT3* promoter region has great potential in the creation of promoter−reporter biosensor systems in genetically engineered bacteria or yeasts for the detection of heavy metal contamination. The development of cheap and simple monitoring systems for toxic metal ions in the environment is increasingly important for the prevention of chronic exposure to these pollutants. The high *FeMT3* promoter inducibility to metals within only 569 bp is an advantage, since smaller-sized promoters are suitable for expression cassette construction and favor multiple integration into host chromosomes (*27*).

Regulating transgene expression for increasing heavy metal tolerance or accumulation in plants could be another possible use for the *FeMT3* promoter region. Employment of the strong constitutively active CaMV35S promoter may have potentially harmful effects due to constant overexpression of the transgene, so inducible promoters are a better choice for this purpose. The finding that the *FeMT3* promoter is much more active in leaves than roots (*14*) is also advantageous, having in mind that only aboveground plant parts are harvested for phytoremediation.

According to our results, *FeMT3* transcription level increased under the influence of various concentrations of Cu and a lower concentration of Cd during short time exposure. However, prolonged treatment with a higher, that is, toxic Cd concentration, resulted in a transcriptional decrease.

The elevation in FeMT3 transcript content could be assigned as moderate in contrast to enormously high promoter inducibility. The reason for this discrepancy could be the existence of additional upstream or downstream regulatory elements or factors that had an impact on transcript stability and longevity.

Increased Tolerance to Heavy Metal Stress of *E. coli* and $Cup1^{\Delta}$ Yeast Cells Overexpressing FeMT3. To test whether the FeMT3 protein could provide protection against cadmium and copper toxicity, it was expressed in CUP1-deficient yeast cells ($cup1^{\Delta}$). As shown in **Figure 5**, the strain expressing FeMT3 was able to complement metal binding properties of the missing cup1 gene and protect cells against the toxic effects of copper and cadmium ions.

A similar test was carried out in prokaryotic system *E. coli* when the metal toxicity protection by recombinant GST-FeMT3 was confirmed. Reduced growth of bacteria expressing only GST in medium supplemented with Cd excluded this protein as metal binding, so the role of “rescuer” belonged to FeMT3.

Increased Tolerance to Heavy Metal Stress of Plant Cells Overexpressing FeMT3. Our results also confirmed the protective role of FeMT3 during heavy metal stress in plant tissue. In this study, we focused on aboveground plant parts as they are easily harvested in phytoremediation, and *FeMT3* was found to be much more expressed in leaves than in roots (*13*). We demonstrated that FeMT3 significantly improved metal stress tolerance in transiently transformed *N. debneyii* leaves. When exposed to $CdCl_2$ or $CuSO_4$, FeMT3 overexpressing leaf regions showed much slower development of tissue damage, as compared with mock-transformed regions. Elevated lipid peroxidation and chlorophyll loss, which are markers of cellular damage due to increased ROS concentrations, have been detected in various plant species upon heavy metal treatment (*6*). We observed a similar trend during Cd/Cu exposure in both FeMT3 expressing and control regions; however, the change of both cellular damage indicators was significantly slower in the presence of overexpressed FeMT3, suggesting its defensive role.

As reported for other types of plant MTs, buckwheat MT3 was also localized in the cytoplasm in both untreated and stressed leaves, implying that its protective function is accomplished in a different manner from that of phytochelatins, which are translocated to the vacuole when complexed with metal ions (*10*, *23*).

Previous investigations, demonstrating a protective role for MTs in plant tissue exposed to various stress conditions, focused mainly on type 1 or 2 MTs (*6*, *23*, *25*, *28*, *29*). A single study demonstrated protective effects of ectopic expression of a MT belonging type 3, in transiently transformed guard cells subjected to $CdCl_2$ (*10*). Another study found GhMT3a from cotton to protect transgenic tobacco but only upon exposure to nonmetal stresses (*9*). On the other hand, TcMT3 from *T. caerulescens* did not enhance tolerance to Cu^{2+}, Zn^{2+}, and Cd^{2+} in transgenic *A. thaliana*, although this MT conferred high Cu tolerance on the yeast cells and exhibited elevated expression in metal-accumulating accessions of *T. caerulescens* (*30*). Perhaps the ability of FeMT3 to confer metal stress tolerance could be attributed to the atypical structure of the FeMT3 C terminus, containing seven Cys residues. However, because data on plant MT protein structures are scarce, it is difficult to assess whether these changes could result in modification of their functional properties.

The protective role of FeMT3 during heavy metal presence was confirmed in prokaryotic and eukaryotic unicellular systems as well as in transgenic higher plants. Taken together with the high promoter inducibility and detected increased transcript level (upon Cd and Cu exposure), these results strongly indicate that FeMT3 plays an important role in buckwheat heavy metal tolerance and hyperaccumulation. Moreover, the *FeMT3* promoter and protein should be considered as new candidates for biotechnological applications, with great potential for use in biosensing and phytoremediation.

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